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(71) Applicant: **Japan Tobacco Inc.**
Tokyo 105-8422 (JP)

(72) Inventors:
- **TAKAKURA, Yoshimitsu**
  Japan Tobacco Inc.
  Toyoda-cho, Iwata-gun Shizuoka 438-0802 (JP)
- **INOUE, Tsuyoshi**
  Japan Tobacco Inc.
  Toyoda-cho, Iwata-gun Shizuoka 438-0802 (JP)
- **SAITO, Hideaki**
  Japan Tobacco Inc.
  Toyoda-cho, Iwata-gun Shizuoka 438-0802 (JP)
- **ITO, Toru**
  Japan Tobacco Inc.
  Toyoda-cho, Iwata-gun Shizuoka 438-0802 (JP)

(74) Representative:
Behnisch, Werner, Dr.
Reinhard-Skuhra-Weise & Partner,
Patentanwälte,
Postfach 44 01 51
80750 München (DE)

(54) NOVEL DNA FRAGMENTS ORDERING GENE EXPRESSION PREDOMINANT IN FLOWER ORGAN

(57) An object of the present invention is to provide a novel DNA sequence having a flower organ-specific promoter activity which makes it possible to express a foreign gene specifically in pistil or lodicule, thus enabling genetic manipulation. The present invention provides a DNA fragment comprising the sequence of positions 1 to 5369 in the nucleotide sequence represented by SEQ ID NO:2 in Sequence Listing, the sequence of positions 3335 to 5108 therein, a part of these sequences or a sequence derived from these sequences by deletion, substitution, insertion or addition of one or more nucleotides and having a promoter activity; and a flower organ-specific promoter sequence which can be identified from among sequences obtained by screening a genomic library of rice or other plants by using as a probe the nucleotide sequence as described above or a part thereof.

EP 0 984 064 A1

Description

TECHNICAL FIELD

[0001] This invention relates to a promoter capable of directing the expression of a foreign gene specifically in flower organs.

PRIOR ART

[0002] Although there have been reported several genes expressed in flower organs including anther-specific genes and pistil-specific ones, the promoter sequences of these genes are poorly understood. Regarding dicotyledons, Mariani et al. (Nature, 347, 737-741, 1990) report analysis on the expression site of a promoter of a tobacco anther tapetal cell-specific gene TA29, while Goldman et al. (The EMBO Journal 13, 2976-2984, 1994) report the isolation of a tobacco pistil stigma-specific gene STIG1 and analysis on the expression site of its promoter. In these reports, it is also stated that male sterile and female sterile tobacco plants were constructed each by linking a bacterial RNase to the promoter and transferring it into plant cells, thus providing an example of artificial manipulations of physiological and morphological characteristics with the use of a tissue-specific promoter. Regarding monocotyledons, on the other hand, there have been reported some cases of the isolation of anther-specific promoters but no pistil-specific promoter so far. For example, JP (Kohyo) HEI 6-504910 reports the isolation of a rice anther-specific gene, its promoter and use thereof, while Tsuchiya et al. (Plant Mol. Biol. 26, 1737-1746, 1994) report analysis on the expression of a rice immature anther tapetal cell-specific promoter.

[0003] Promoters exhibiting expression specifically in flower organs are desired in order to artificially improve the morphology of plant flower organs, in particular, germ organs or physiological phenomena or to analyze functions of various genes in flower organs. In monocotyledons which represent major cereals, however, few genes expressed exclusively in flower organs have been isolated hitherto. In particular, there has been reported no promoter sequence showing predominant expression in pistil which is the female germ organ or lodicule which regulates flowering.

DISCLOSURE OF THE INVENTION

[0004] An object of the present invention is to provide a novel DNA sequence having a flower organ-specific promoter activity which makes it possible to express a foreign gene specifically in pistil or lodicule, thus enabling genetic manipulations which were impossible in the prior art particularly in monocotyledons.

[0005] To achieve the above-described object, the present inventors have conducted extensive research and, as a result, succeeded in the isolation and identification of a clone showing flower organ-specific expression through a differential screening of a paddy rice pistil cDNA library with the use of a pistil probe and a leaf probe, thus completing the present invention.

[0006] In the first aspect, the present invention provides a DNA fragment comprising the sequence of positions 3335 to 5108 in the nucleotide sequence represented by SEQ ID NO:2 in Sequence Listing, a part of said sequence or a sequence derived from said sequence by deletion, substitution, insertion or addition of one or more nucleotides and having a promoter activity.

[0007] In a preferred embodiment of the present invention, it provides a DNA fragment wherein a part of the sequence of positions 3335 to 5108 in the nucleotide sequence represented by SEQ ID NO:2 is located downstream of the point of at least 500 nucleotides upstream of the transcription initiation points (the nucleotides of positions 4995 to 4997 in the nucleotide sequence represented by SEQ ID NO:2).

[0008] In another preferred embodiment of the present invention, a DNA fragment is provided wherein a part of the sequence of positions 3335 to 5108 in the nucleotide sequence represented by SEQ ID NO:2 is located in the region upstream of the first initiation codon (the nucleotides of positions 5016 to 5018 in the nucleotide sequence represented by SEQ ID NO:2).

[0009] In another preferred embodiment of the present invention, a DNA fragment is provided wherein the region upstream of the initiation codon is located downstream of the point of at least 500 nucleotides upstream of the transcription initiation points.

[0010] In another preferred embodiment of the present invention, a DNA fragment is provided wherein a part of the sequence of positions 3335 to 5108 in the nucleotide sequence represented by SEQ ID NO:2 is located in the region upstream of transcription initiation points (the nucleotides of positions 4995 to 4997 in the nucleotide sequence represented by SEQ ID NO:2).

[0011] In another preferred embodiment of the present invention, a DNA fragment is provided wherein the region upstream of the transcription initiation points is the region of at least 500 nucleotides upstream of the transcription initiation points.

[0012] In another preferred embodiment of the present invention, a DNA fragment is provided comprising the sequence of positions 3335 to 5108 in the nucleotide sequence represented by SEQ ID NO:2 in Sequence Listing or a sequence derived from the above sequence by deletion, substitution, insertion or addition of one or more nucleotides and having a promoter activity.

[0013] In the second aspect, the present invention provides a DNA fragment comprising the sequence of positions 1 to 5369 in the nucleotide sequence represented by SEQ ID NO:2 in Sequence Listing, a part of said sequence or a sequence derived from said sequence by deletion, substitution, insertion or addition of one or more nucleotides and having a promoter activity.

[0014] In a preferred embodiment of the present invention, a DNA fragment is provided wherein a part of the sequence of positions 1 to 5369 in the nucleotide sequence represented by SEQ ID NO:2 is located downstream of the HindIII site (the nucleotides of positions 3335 to 3340 in the nucleotide sequence represented by SEQ ID NO:2).

[0015] In another preferred embodiment of the present invention, a DNA fragment is provided wherein a part of the sequence of positions 1 to 5369 in the nucleotide sequence represented by SEQ ID NO:2 is located downstream of the point of at least 500 nucleotides upstream of transcription initiation points (the nucleotides of positions 4995 to 4997 in the nucleotide sequence represented by SEQ ID NO:2).

[0016] In another preferred embodiment of the present invention, a DNA fragment is provided wherein a part of the sequence of positions 1 to 5369 in the nucleotide sequence represented by SEQ ID NO:2 is located upstream of the third Bg1II site (the nucleotides of positions 5103 to 5108 in the nucleotide sequence represented by SEQ ID NO:2).

[0017] In another preferred embodiment of the present invention, a DNA fragment is provided wherein a part of the sequence of positions 1 to 5369 in the nucleotide sequence represented by SEQ ID NO:2 is located in the region upstream of the first initiation codon (the nucleotides of positions 5016 to 5018 in the nucleotide sequence represented by SEQ ID NO:2).

[0018] In another preferred embodiment of the present invention, a DNA fragment is provided wherein a part of the sequence of positions 1 to 5369 in the nucleotide sequence represented by SEQ ID NO:2 is located upstream of the transcription initiation points (the nucleotides of positions 4995 to 4997 in the nucleotide sequence represented by SEQ ID NO:2).

[0019] In another preferred embodiment of the present invention, a DNA fragment is provided comprising the sequence of positions 1 to 5369 in the nucleotide sequence represented by SEQ ID NO:2 in Sequence Listing or a sequence derived from said sequence by deletion, substitution, insertion or addition of one or more nucleotides and having a promoter activity.

[0020] In the third aspect, the present invention provides a chimeric DNA sequence comprising a DNA fragment of the present invention having a promoter activity as described above and a desired structural gene under the regulation of the same.

[0021] In the fourth aspect, the present invention provides a transformation vector having a chimeric DNA sequence according to the present invention as described above.

[0022] In the fifth aspect, the present invention provides a DNA fragment having a flower organ-specific promoter activity which is hybridizable with the sequence of positions 1 to 5369 in the nucleotide sequence represented by SEQ ID NO:2 in Sequence Listing or a part of the sequence having a flower organ-specific promoter activity. In a preferred embodiment of the present invention, hybridization is performed under conditions with a moderate hybridization intensity.

[0023] In the sixth aspect of the present invention, a flower organ-specific promoter sequence is provided which can be identified on the basis of a DNA sequence obtained by screening a genomic DNA library of rice or other plants by using as a probe the nucleotide sequence represented by SEQ ID NO:1 in Sequence Listing or a part of said sequence. In a preferred embodiment of the present invention, screening is performed under conditions with a moderate hybridization intensity.

[0024] In the seventh aspect of the present invention, a DNA fragment is provided comprising a sequence having at least 15 consecutive nucleotides in the sequence from positions 22 to 1278 of the sequence represented by SEQ ID NO:1 in Sequence Listing or a nucleotide sequence complementary to said sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025]

Fig. 1 is a photograph showing the results of Northern analysis on RPC213.
Fig. 2 consists of photographs showing the results of RT-PCR analysis on RPC213.
Fig. 3 is a drawing illustrating comparison of the restriction maps of RPC213 and RPG106.
Fig. 4 is a drawing illustrating the nucleotide sequence around the transcription initiation points.
Fig. 5 is a photograph showing the results of primer extension analysis.

Fig. 6 is a model view showing a procedure for constructing vectors for analyzing the promoter expression.

Fig. 7 consists of graphs showing the results of the GUS-analysis with regard to expression sites of 213 promoter.

Fig. 8 consists of photographs showing exemplary results of the GUS-analysis with regard to expression sites of 213 promoter.

PREFERRED MODE FOR CARRYING OUT THE INVENTION

[0026] Now, the present invention will be described in greater detail.

[0027] As described above, one of the inventions produced by the present inventors relates to a DNA fragment comprising the sequence of positions 1 to 5369 in the nucleotide sequence represented by SEQ ID NO:2 in Sequence Listing, the sequence of positions 3335 to 5108 therein, a part of said sequences or a sequence derived from said sequences by deletion, substitution, insertion or addition of one or more nucleotides and having a promoter activity.

[0028] The promoter sequence of the present invention, namely, the sequence comprising the nucleotides of positions 1 to 5369 in the sequence represented by SEQ ID NO:2 has no homology to any known promoter sequence. Thus, this sequence is considered to be a novel promoter sequence.

[0029] The DNA fragment of the present invention has a promoter activity specific to flower organs. The term "flower organ-specific promoter activity" as used herein means that the expression of the promoter activity of the DNA fragment of the present invention in flower organs (immature pistil in earing period, mature pistil in flowering period, lodicule, and palea and lemma) is more prominent than in other organs. In the reverse transcription PCR experiment performed in Examples as will be shown hereinafter, the expression levels in the organs other than these flower organs were less than 1/100 of the expression level in immature pistil. In this case, examination was made of anther in flowering period, leaf and root about 1 month after sowing, immature seed 1 to 2 weeks after fertilization, germinating seed, and callus, in addition to the three flower organs as described above (Figs. 1 and 2, Table 1).

[0030] The nucleotide sequence represented by SEQ ID NO:2 in Sequence Listing has the following characteristics.

1. It has 3 consecutive transcription initiation points consisting of nucleotides, from upstream, C (cytosine), A (adenine) and A (adenine) (i.e., nucleotides of positions 4995 to 4997 in the sequence represented by SEQ ID NO:2).
2. There is a TATA box-like sequence (5'-TATAAA-3') (nucleotides of positions 4964 to 4969 in the sequence represented by SEQ ID NO:2) (Corden et al. Science 209, 1406-1414, 1980) 31 bp upstream of the most upstream transcription initiation point C (cytosine).
3. The first initiation codon ATG (nucleotides of positions 5016 to 5018 in the sequence represented by SEQ ID NO:2) is located 21 bp downstream of the most upstream transcription initiation point C (cytosine).
4. The second ATG is located in the same reading frame 273 bp downstream of the first initiation codon, while an intron sequence of 81 bp is located between them.

[0031] In the present invention, regions upstream of the presumed structural gene regions (although we do not exclude a possibly that these regions may include some part of the 5'-terminus region of the structural gene), namely, the sequence consisting of the nucleotides of positions 1 to 5369 and the sequence consisting of the nucleotides of positions 3335 to 5108 in the nucleotide sequence represented by SEQ ID NO:2 are specified as promoter sequences. Moreover, sequences comprising a part of these sequences fall within the scope of the present invention, so long as they have promoter activity. For example, it is expected that the region of positions 1 to 4994 and the region of positions 3335 to 4994 have a promoter activity, since the transcription initiation point is located at position 4995 as described above.

[0032] Moreover, said latter sequence consisting of the nucleotides of positions 3335 to 5108 in SEQ ID NO:2 was specified as a promoter sequence, since a HindIII cleavage site is located at position 3335 by chance. Therefore, it is well anticipated that a sequence starting from a nucleotide somewhat downstream will have the promoter activity too. This is so because a number of reports indicate that the tissue- or time-specificity or inducibility of most plant promoters is sustainedlly contained in the region of 0.3 to 0.4 kbp which precedes the transcription initiation point. In the promoter of type II glutelin gene of rice, for example, the tissue- and time-specific expression in albumen was fully achieved by a 441 bp fragment of the upstream region of the transcription initiation point (Takaiwa et al. Plant Mol. Biol. 16:49-58, 1991). In the promoter of self-incompatibility-related gene SLG13 of *Brassia oleracea*, the 411 bp upstream region of the transcription initiation point directed the expression in pistil and pollen (Dzelkalns et al. The Plant Cell 5: 855-863, 1993). In the promoter of anionic peroxidase gene of tomato, the organ-specificity as well as the pathogen and wound-inducibility were determined by the 358 bp upstream region of the transcription initiation point (Mohan et at. Plant Mol. Biol. 22: 475-490, 1993). Thus, it is observed in a number of promoters that a part of the reported nucleotide sequence maintains the full function as a promoter, in particular, the specificity, if only said part is the region located within several hundred bp upstream of the transcription initiation point.

[0033] Accordingly, any sequence comprising a DNA fragment from the region within several hundred bp, preferably

about 500 bp, upstream of the transcription initiation points and having the flower organ-specificity characterized in the present invention is included in the scope of the present invention. For example, if a region within several hundred bp upstream of the transcription initiation point or a region containing the same is easily isolated from rice genome by PCR with the use of primers designed based on the nucleotide sequence of the present invention and the region exhibits the flower organ-specificity inherent to the promoter of the present invention, then the shorter promoter sequence is included in the scope of the present invention.

[0034] The DNA fragment of the present invention can be obtained by, for example, starting with rice by the methods as will be described in the following Examples. Alternatively, it can be easily prepared by PCR with the use of rice genome as a template by using as primers a pair of oligonucleotides corresponding respectively to both termini of the DNA fragment of the present invention the nucleotide sequence of which has been clarified. In order to determine whether or not the sequence has flower organ-specificity, a chimera gene can be constructed by ligating β-glucuronidase (GUS) gene to the promoter sequence and the resultant chimera gene is introduced into rice plant to thereby confirm the expression sites.

[0035] The present invention further includes in its scope DNA fragments having a sequence derived from these sequences by deletion, substitution, insertion or addition of one or more nucleotides and showing the promoter activity.

[0036] It is well known that when a nucleotide sequence of a DNA having a physiological activity is slightly modified by substitution, deletion, addition or insertion of one or more nucleotides in the nucleotide sequence thereof, the physiological activity of the DNA is maintained in general. Therefore, the present invention includes within the scope thereof DNA sequences derived from the above mentioned promoter sequence by such slight modification and having the promoter activity. That is to say, the sequence consisting of the nucleotides of positions 1 to 5369 in the nucleotide sequence represented by SEQ ID NO:2 in Sequence Listing, the sequence consisting of the nucleotides of positions 3335 to 5108 therein, parts of these sequences having the promoter activity (for example, those consisting of a several hundred bp region upstream of the transcription initiation points), and DNA sequences derived therefrom by deletion, substitution, insertion or addition of a small number of nucleotides and having the promoter activity are all intended to be included in the scope of the present invention.

[0037] Similarly, the sequence consisting of the nucleotides of positions 1 to 4994 in the nucleotide sequence represented by SEQ ID NO:2 in Sequence Listing, the sequence consisting of the nucleotides of positions 3335 to 4994 therein and DNA sequences derived therefrom by deletion, substitution, insertion or addition of a small number of nucleotides and having the promoter activity are included in the scope of the present invention.

[0038] The addition, insertion, deletion or substitution of nucleotides can be carried out by, for example, site-directed mutagenesis (see, for example, Nucl. Acids Res. 10:6487-6500, 1982) which is a well-known technique. The expression "one or more nucleotides" as used herein means nucleotides in such a number as to allow addition, insertion, deletion or substitution by the site-directed mutagenesis method.

[0039] Site-directed mutagenesis can be performed in the following manner with the use of, for example, a synthetic oligonucleotide primer which is complementary to the single-stranded phage DNA to be mutated except a specific discordance, i.e., the desired mutation. Namely, a complementary strand is synthesized by a phage with the use of the above-mentioned oligonucleotide as a primer. Next, a host bacterium carrying the phage is transformed by the double-stranded DNA thus obtained. The culture of the transformed bacterium is then plated onto agar and plaques containing the phage from a single cell are formed. Thus theoretically 50% of the newly formed colonies will contain the phage carrying the mutation in the single strand while the remaining 50% of the colonies have the original sequence. The plaques thus obtained are hybridized with a synthetic probe having been treated with kinase at such a temperature as to allow the hybridization of the plaques coinciding with the DNA having the desired mutation as described above but not with those having the original strands. Then the plaques hybridized with the probe are picked up and cultured to subsequently recover the DNA.

[0040] In addition to the above site-directed mutagenesis method, nucleotide(s) can be substituted, deleted, added or inserted into the promoter sequence while maintaining its activity by treating the gene with a mutagen or by selectively cleaving the gene and then deleting, adding or substituting the desired nucleotide(s) followed by ligation.

[0041] Also, the substitution, deletion, addition or insertion of specific nucleotide(s) may be conducted by the site-directed mutagenesis with the use of the PCR method (Mikaelian et al. Nucl. Acids Res. 20:376, 1992) or the random nucleotide substitution technique (Zhou at al. Nucl. Acids Res. 19:6052, 1991) by taking advantage of the low fidelity of Tag DNA polymerase.

[0042] Now, another invention found by the present inventors will be illustrated.

[0043] This invention relates to a flower organ-specific promoter sequence which can be identified from among sequences obtained by screening a genomic library of rice or other plants by using as a probe the nucleotide sequence represented by SEQ ID NO:1 in Sequence Listing or a part of said sequence.

[0044] The nucleotide sequence represented by SEQ ID NO:1 can be obtained by the differential screening method with the use of rice (IR24) as will be described in Examples hereinafter. Alternatively, it can be easily prepared by the PCR method with the use of a rice flower organ-derived cDNA or rice genome as a template by using as primers a pair

of oligonucleotides corresponding respectively to both termini of the DNA fragment of the present invention the nucleotide sequence of which has been clarified.

[0045] Either the whole nucleotide sequence or a part thereof may be used as a probe.

[0046] The genome library can be constructed by using rice green leaf by, for example, the method which will be described in detail in Examples hereinafter, though the present invention is not limited thereto. A genomic fragment containing the promoter is prepared from the thus obtained library by using the above-described probe and thus the promoter sequence is identified. In order to determine whether or not the sequence has flower organ-specificity, a chimera gene can be constructed by ligating β-glucuronidase (GUS) gene to the promoter sequence and the resultant chimera gene is introduced into a desired plant to confirm the expression sites.

[0047] The promoter sequence thus obtained should have flower organ-specificity of such an extent comparable (at least being predominant in any flower organ) to the specificity as will be described in Example 3(2) hereinafter.

[0048] Finally, a probe for detecting a flower organ-specific promoter, which is another aspect of the present invention, will be illustrated.

[0049] The probe according to the present invention comprises a DNA fragment comprising a sequence having at least 15 consecutive nucleotides in the sequence from positions 22 to 1278 of the sequence represented by SEQ ID NO:1 in Sequence Listing or a nucleotide sequence complementary to said sequence. It is highly possible that this sequence of positions 22 to 1278 in the sequence represented by SEQ ID NO:1 or a nucleotide sequence highly homologous thereto will undergo the flower organ-specific expression as described above. By using this sequence or a part thereof as a probe in examining plant genomic DNA, therefore, a novel flower organ-specific promoter occurring in rice or other plants can be found out.

[0050] The probe is designed based on the above-mentioned sequence. It preferably has at least 15 consecutive nucleotides. There is no particular upper limit of its length up to the full length of the sequence as described above. The present invention also includes within the scope thereof sequences which are derived from a DNA fragment selected from those described above by addition, deletion, insertion or substitution of one or more nucleotides while being hybridizable with the above sequence or a sequence highly homologous thereto. The addition, deletion, insertion or substitution can be performed by the same methods as described above regarding the flower organ-specific promoter according to the present invention.

[0051] The probe of the present invention can be prepared by cleaving the DNA fragment represented by SEQ ID NO:1 in Sequence Listing, which is obtained by the method as will be described in detail in Examples hereinafter, with appropriate restriction enzymes. Alternatively, it can be prepared by the PCR method with the use of a sample comprising this sequence. It is also possible to synthesize a single-stranded DNA serving as a probe by a conventional method with the use of a marketed DNA synthesizer (for example, one manufactured by Perkin Elmer).

[0052] The probe according to the present invention can be labeled by a conventional method with, for example, a radioisotope. For example, the random priming labeling method is employed to label the probe with ^{32}P, while the 5'-terminal labeling method with the use of phosphorylating enzyme is employed when a synthetic oligomer is used.

[0053] When the probe of the present invention is used, hybridization can be performed by a conventional method. In general, hybridization is carried out under conditions giving a moderate hybridization intensity, i.e., performing the hybridization and washing at room temperature to 50°C at an appropriate ionic strength (for example, 0-50% formamide, 6 x SSC, 1 x Denhart's solution, etc.). The probe of the present invention is used in a genome library of the plant to be treated and then the genomic DNA of the plant thus hybridized is isolated. Next, the upstream region of this gene is identified to thereby give a novel flower organ-specific promoter.

[0054] The flower organ-specific promoter of the present invention is a novel flower organ-specific promoter sequence which makes it possible to genetically manipulate and improve pistil and lodicule. This was previously impossible particularly in monocotyledons. Thus, the promoter is useful for, e.g., the following purposes.

1) Improvement in fertilizability of female germ organs by ligating a structural gene capable of enhancing tolerance to stress (such as cold weather, drought, hot, etc.) to the promoter sequence of the present invention or a part thereof.

2) Creation of female sterile plants by ligating a structural gene capable of inducing sterility to the promoter sequence of the present invention or a part thereof.

3) Flower organ-specific proliferation or enlargement by ligating a structural gene capable of promoting proliferation or division of plant cells to the promoter sequence of the present invention or a part thereof.

4) Genetic regulation of flowering by means of the expression of the promoter of the present invention in lodicule.

5) Providing the whole flower organs or a particular site thereof (for example, pistil) with an improved tolerance by ligating a gene capable of inducing an improved tolerance to herbicides or diseases to the promoter sequence of the present invention or a part thereof.

[0055] To further illustrate the present invention in greater detail, and not by way of limitation, the following Examples

are given.

EXAMPLES

Example 1: Isolation of flower organ-specific cDNA

[0056] Paddy rice varieties "Tsukinohikari" and "IR24" were grown in a greenhouse and subjected to the following experiments.

(1) Extraction of RNA

[0057] The leaf, immature pistil, mature pistil, anther, lodicule, palea and lemma, immature seed, germinating seed, root, callus and immature spikelet (4.5 to 6.0 mm in length) of "IR24" were collected, immediately frozen in liquid nitrogen and then stored at -80°C. The total RNA was extracted from these tissues by the SDS-polyphenol method (Watanabe and Price, Proc. Natl. Acad. Sci. USA, 79, 6304-6308, 1982) except that β-mercaptoethanol was added as an antioxidant to the extraction buffer to give a final concentration of 10% (V/V). The tissues to be used in the reverse transcription PCR experiment were treated with DNase I (FPLC pure, manufactured by Pharmacia) in the presence of RNase inhibitor (RNAguard, manufactured by Pharmacia), rather than being subjected to lithium chloride precipitation, so as to minimize the contamination with any trace amount of DNA. 0.375 μg/μl of the total nucleic acid and 1.75 U/μl of RNase inhibitor were added in a buffer (40 mM Tris-Cl pH 7.5, 6mM $MgCl_2$) and 0.375 U/μl of DNase I (each expressed in the final concentration) was added thereto. After maintaining at 37°C for 10 to 30 minutes, DNase I was inactivated by extraction with phenol/chloroform.

[0058] The leaf and root [expressed in root (soil) in Fig. 1] were collected from a plant grown for 1 month in a greenhouse after sowing. The immature pistil was collected from a plant 1 to 2 weeks before earing. The mature pistil, anther, lodicule and palea and lemma were collected from a plant immediately to several days before flowering. The immature seed was collected from a plant 1 to 2 weeks after flowering. The germinating seed and root were obtained from a plant aseptically grown on an N6 medium (Chu et al. Scientia Sinica, 18, 659-668, 1975) respectively for 1 and 3 weeks after sowing. The callus was induced from a seed in an N6 solid medium containing 2 mg/l of 2,4-D and then cultured before use in a liquid medium of the same composition under shaking for 3 weeks. The total RNA of the pistil and leaf was purified to provide polyA+RNA by using Oligotex-dT30 super (manufactured by Takara Shuzo Co., Ltd.) in accordance with the manufacturer's instructions.

(2) Construction of pistil cDNA library

[0059] About 1 μg of polyA+RNA isolated and purified from pistil was employed as a template to synthesize the cDNA by using ZAP-cDNA Synthesis Kit (manufactured by STRATAGENE). The determination of ^{32}P uptake ratio indicated that about 55 ng of the first strand cDNA of the pistil was reversely transcribed by the olig-dT priming, and about 72 ng of the second strand cDNA was synthesized directly from the first strand. The cDNA was connected to an EcoRI adapter in accordance with the manufacturer's instructions, digested with XhoI and then ligated into vector UniZAP XR. Next, the phage DNA was packaged into phage particles by using Giga pack Gold packaging extract (manufactured by STRATAGENE). The phage was transfected into *E. coli* PLK-F' host cells, which were then inoculated on a plate. The library size of the pistil cDNA library was calculated as 3×10^6 pfu.

(3) Differential screening

[0060] Differential screening was carried out basically in accordance with the method of Gasser et al. (The Plant Cell 1, 15-24, 1989). About 2,000 pfu of the phage from the pistil cDNA library was infected into *E. coli* PKL-F' cells and the cells were plated on square Petri dishes (14 x 10 cm). For each plate, a replica filter was prepared with the use of a nylon membrane filter Hybond-N+ (manufactured by Amersham) and the filter was treated in accordance with the manufacturer's instructions. As the probes for hybridization, use was made of single-stranded cDNA synthesized from about 100 ng of the polyA+RNA (or about 2 μg of the total RNA) of pistil and leaf. To 2 μg of an RNA solution, 0.5 mM of d(ATG)TP, 10 mM of DTT and 1 x M-MuLV buffer (manufactured by BRL) were added. Next, 30 ng/μl of Random DNA Hexamer (manufactured by Pharmacia) [or 80 ng/μl of Oligo dT Primer (manufactured by Amersham)] was added thereto (each expressed in the final concentration). After dissociating the secondary structure of the RNA by heating at 65°C for 5 minutes, the primer was annealed at room temperature. After further adding 1.5 U/μl of RNase inhibitor (RNA guard manufactured by Pharmacia), 10 U/μl of reverse transcriptase M-MuLV (manufactured by BRL) and 4 μCi/μl of [α-^{32}P]dCTP (each expressed in the final concentration), the liquid reaction mixture of 20 μl in total was incubated at 37°C for 1 hour.

[0061] Subsequently, dCTP (RI-unlabeled) was further added to give a final concentration of 0.5 mM and the reaction was continued for 30 minutes. The labeled DNA probe were purified by using Quick Spin Column G-50 Sephadex (manufactured by BOEHRINGER MANNHEIM). The probes were single-stranded by adding an equivalent amount of 2 N NaOH (final concentration: 1 N). The filter was first treated in a pre-hybridization buffer (0.25 M Na_2HPO_4, pH 7.2, 7% SDS, 1 mM EDTA, 1 x Denhart's solution) at 68°C for 10 minutes. Then the single-stranded probes (final concentration: 0.2-0.3 x 10^7 cpm/ml) and carrier DNA (a mixture of 0.1 mg/ml of salmon sperm DNA, 0.1 μg/ml of λDNA, 0.1 μg/ml of rice DNA) were added thereto and hybridization was performed at 68°C overnight (16 to 24 hours). The filter was washed in the buffer (20 mM Na_2HPO_4, pH 7.2, 1% SDS, 1 mM EDTA) at room temperature twice and at 68°C twice each for 15 minutes. Next, this filter was exposed to Kodak X-Omat Film at -70°C for 4 to 5 days.

[0062] When about 30,000 plaques were examined, 198 plaques showing intense hybridization signals with the pistil probe but only weak signals with the leaf probe were selected by the primary screening. 152 clones among them were subjected to the secondary screening. To avoid intense background of plaque hybridization in this step and to efficiently perform screening, the following method was employed. First, the plaques selected by the primary screening were stored in 200 μl of SM buffer (0.1 M NaCl, 7 mM $MgSO_4$, 50 mM Tris-CL pH 7.5, 0.01% gelatin) containing one drop of chloroform at 4°C. Then the thus stored liquid was diluted and the phage was plated so as to give a considerably low plaque density (10 to 100 pfu/plate). A plaque separated from others was isolated and stored in the same buffer. From this liquid, a plating lysate containing the phage at a high concentration was prepared and *in vivo* excision was performed in accordance with the instructions attached to ZAP cDNA Synthesis Kit. Thus a plasmid [pBluescriptSK(-)] was prepared from the phage genome.

[0063] Then it was digested with restriction enzymes EcoRI and XhoI (manufactured by Takara Shuzo Co., Ltd.) and thus a cDNA insert was isolated and purified. This cDNA insert was fractionated by electrophoresing on a 0.8% agarose gel and blotted onto a nylon membrane filter HybondN+. Then differential hybridization was carried out with the use of pistil and leaf probes as well as single-stranded cDNA probes synthesized by using Oligo dT Primer from the total RNA of anther, germinating seed, root, callus or immature seed. As a result, 6 cDNA clones which were hybridizable with the pistil probe but little with other probes were obtained. Among these clones, one having an insert cDNA of about 1.5 kb was named "RPC213" and employed in the subsequent experiments.

(4) Analysis on organ-specific expression of cDNA clones

1) Northern hybridization analysis

[0064] The cDNA clone "RPC213" screened in the above 3) was subjected to Northern hybridization to examine the expression patterns and expression levels in various organs. Filters were prepared in the following manner.

[0065] First, the secondary structure of the total RNA (20 μg) from each of the organs described in the above 1) was dissociated in accordance with the method of Sambrook et al. (Molecular Cloning, 1982) with the use of deionized Glyoxal and DMSO and then fractionated in a 1% agarose gel. Next, the RNA was blotted onto a nylon membrane Gene Screen Plus (manufactured by DU PONT) by the conventional method. After drying in vacuo at 80°C for 1 hour, the filter was boiled in 20 mM Tris-Cl (pH 8.0) for 5 minutes to thereby remove Glyoxal therefrom. As a probe, the 1.5 kb EcoRI-XhoI fragment of the above-mentioned cDNA was RI-labeled by using Multiprime Labeling System (manufactured by Amersham). Pre-hybridization and hybridization were carried out in accordance with the manufacturer's instructions attached to the filter. The filters were washed with 2 x SSC, 1% SDS and 0.2 x SSC, 1% SDS at room temperature each for 5 minutes, then with 0.16 x SSC, 1% SDS at 65°C for 15 minutes twice, and then with 2 x SSC at room temperature for 1 minute. Subsequently, the filters were exposed to Kodak X-Omat Film at -70°C overnight.

[0066] As a result, an intense hybridization signal was observed in the lane of mature pistil, weak signals were observed in the lanes of palea and lemma and callus, and very weak signals were observed in the lanes of leaf, anther and immature seed, while other lanes showed no signal, as Fig. 1 shows. Thus, it was clarified by the results of the Northern analysis that the isolated clone relatively strongly expressed in mature pistil and weakly in palea and lemma, and callus but scarcely in leaf, anther and immature seed. The size of the transcripts was estimated to be about 1.6 kb.

2) Reverse transcription PCR (RT-PCR) analysis

[0067] To analyze the organ-specific expression of the cDNA clone at a higher sensitivity, reverse transcription PCR was carried out by using RNA of various rice organs as templates. By using GENESIS 200 Fluorescence Sequencer (manufactured by DU PONT), the nucleotide sequence of the cDNA inserted into the plasmid pBluescript SK(-) was first partly determined. In accordance with the manufacturer's instructions attached to the Sequencer, T7 DNA polymerase reaction was performed by using M13 and M4 primers (manufactured by Takara Shuzo Co., Ltd.) followed by electrophoresis on a 6% acrylamide gel. Then, the nucleotide sequence was determined from both of the 5'-(EcoRI) and 3'-(XhoI) sides. Based on the DNA nucleotide sequence of about 400 nucleotides (mRNA sense strand) in the 3'-side, the

following primers:

213S; 5'-CGCTATGGCCCGTTTCAGCT-3' (SEQ ID NO:3), and
213AS; 5'-GTCGTCCTGCCGCTTCATTAC-3' (SEQ ID NO:4)

were synthesized with DNA Synthesizer (manufactured by ABI), purified by OPC Cartilage (manufactured by ABI) and employed in the reverse transcription PCR experiment. It was expected that a product of about 250 bp would be amplified with these primers.

[0068] 10 μl of the total RNA of each of the above-mentioned organs was mixed with 500 ng of Oligo dT15 Primer (manufactured by Amersham) and the secondary structure thereof was dissociated by treating in 55 μl of the liquid reaction mixture at 70°C for 10 minutes. After quenching on ice, the mixture was maintained in 100 μl comprising 1 x 1st strand buffer (manufactured by BRL), 0.5 mM of dNTPmix, 10 mM of DTT, 2 U/μl of RNase inhibitor (RNAguard, manufactured by Pharmacia) and 10 U/μl of reverse transcriptase (Superscript: manufactured by BRL) (each expressed in the final concentration) at 37°C for 60 minutes. Next, it was treated at 95°C for 5 minutes to dissociate the RNA-cDNA hybrid and then cooled on ice. The cDNA concentration of this solution was assumed to be 100 ng/μl. Next, the synthesized cDNA of each organ was diluted in 4 series (100 ng/μl, 10 ng/μl, 1 ng/μl, 0.1 ng/μl) and employed as a template in PCR.

[0069] PCR was carried out under the following conditions. 1 μl of the cDNA dilution was mixed with 0.5 pmole/μl of primer, 0.2 mM dNTP, 1 x PCR buffer and 0.05 U Tag Polymerase (manufactured by Takara Shuzo) (each expressed in the final concentration) to give 20 μl of a reaction mixture. By using Gene Amp 9600 (manufactured by Perkin Elmer), the reaction mixture was subjected to PCR consisting of 3 minutes at 94°C for 1 cycle, 0.5 minutes at 94°C, 1 minute at 60°C and 1 minute at 72°C for 30 cycles and 6 minutes at 72°C for 1 cycle. The PCR product was electrophoresed on an agarose gel, stained with ethidium bromide and then photographed. Bands were compared with each other in density, and 2 samples showing the same density were estimated to contain the cDNA originating in the above-mentioned gene in the same amount.

[0070] It was preliminarily confirmed, by using plasmid clones, that the product of the expected molecular weight could be amplified with the primers for RPC213 gene. When reverse transcription PCR was performed by using this primer and 100 ng of cDNA as a template, dense bands of the PCR products were observed in mature pistil, palea and lemma and callus, faint bands were observed in anther and immature seed and exclusively faint bands were observed in leaf, germinating seed and root, as shown in Fig. 2A. Among these organs, mature pistil and palea and lemma showed the PCR product after diluting the template cDNA to 1 ng, while callus, anther and immature seed showed the product only until the template cDNA was reduced to 10 ng. Leaf, germinating seed and root showed no PCR product, when the template cDNA was diluted to be less than 100 ng. When the expression level in mature pistil was taken as 1, it was estimated based on the band density that the expression level in palea and lemma was about 1 to 1/10, those in anther, immature seed and callus were about 1/10 and those in other organs were about 1/100.

[0071] Next, differences in expression levels depending on flower organ sites and development stages were analyzed. cDNAs prepared from whole mature pistil, stigma of mature pistil, ovary of mature pistil, whole immature pistil and lodicule were employed as templates. Also, use was made of leaf cDNA and plasmid DNA as controls. Then PCR was carried out with the use of RPC213-specific primers. As a result, when 10 ng of cDNA was employed as a template, the PCR product was detected in all of the organs other than leaf, as shown in Fig. 2B. Among these organs, immature pistil, stigma and lodicule showed the PCR product even though the template was reduced to 0.1 ng, while mature pistil and its ovary showed the PCR product only until the template was reduced to 1 ng. When the RPC213 expression level in the whole mature pistil was taken as 1, it was estimated based on the above results that the expression level in immature pistil, stigma and lodicule were about 10 and that in ovary was about 1. Namely, the results of the reverse transcription PCR indicate that the RPC213 gene is strongly and predominantly expressed in immature pistil, mature pistil stigma and lodicule but weakly in mature pistil ovary and palea and lemma and scarcely in other organs.

[0072] Table 1 summarizes the results of 1) the Northern analysis and the results of 2) the RT-PCR.

TABLE 1

Table 1: Analysis on RPC213 gene expression

Organ	mature pistil	stigma	ovary	immature pistil	lodicule	palea/ lemma	anther	Immature seed	germinating seed	leaf	root	root (soil)	callus
Northern Analysis	++	NT	NT	NT	NT	+	-	±	-	±	-	-	+
RT-PCR	1	10	1	10	10	1~0.1	0.1	0.1	0.01	0.01	0.01	NT	0.1

++: strong expression; +: weak expression; ±: little expression; -: no expression; and NT: not analyzed.

RT-PCR: expressed in relative value determined by taking the expression level in mature pistil as 1.

(5) Determination of the nucleotide sequence of RPC213

[0073] The entire nucleotide sequence of the cDNA clone RPC213 (about 1.5 kb), which is expressed specifically in flower organs, was determined in the following manner with the use of Fluorescence Sequencer (Model 373A, manufactured by Applied Biosystems). Based on the nucleotide sequence information obtained by using the M13 primers (manufactured by Takara Shuzo Co., Ltd.) described above, primers were synthesized and the nucleotide sequence in an undecoded region was determined. By repeating this primer walking procedure, the nucleotide sequence of RPC213 having 1496 bp in total was determined. The reading frame with the largest ORF was identified by the ORF analysis. In this reading frame, polyA signal-like sequences (Heidecker and Messing, Annu. Rev. Plant Physiol. 37, 439-466, 1986) were located about 70 bp and 90 bp downstream of the termination codon TGA. The entire nucleotide sequence of RPC213 is represented by SEQ ID NO:1 in Sequence Listing, though the nucleotide sequence of SEQ ID NO:1 has 1524 bp including a 28 bp segment following the transcription initiation points which was added by reference to the nucleotide sequence of genome clone as will be described hereinafter.

[0074] The sequence represented by SEQ ID NO:1 has the following characteristics.

nt1, nt2, nt3 : transcription initiation points of the RPC213 gene determined by the primer extension method.
nt22-nt24 : the first potential initiation codon of the RPC213 gene.
nt295-nt297 : the second potential initiation codon of the RPC213 gene.
nt1276-nt1278 : the termination codon of the RPC213 gene.
nt1343-nt1348, nt1365-nt1370 : PolyA addition signals.
nt1507-nt1524 : PolyA.

Example 2: Isolation of promoter

(1) Construction of genomic library

[0075] Genomic DNA was isolated by the SDS-phenol method and purified by the lithium chloride precipitation method from "IR24" rice leaves about 2 months after sowing. As a preliminary test, the DNA was first partly digested with a restriction enzyme MboI (manufactured by Takara Shuzo Co., Ltd.) to determine the digestion conditions which would allow the formation of as many fragments of 16 to 23 kb in apparent size as possible. Next, the genomic DNA was digested under the so determined reaction conditions and subjected to sucrose density gradient centrifugation. Sucrose was dissolved in a buffer (20 mM Tris-HCl pH 8.0, 1 mM EDTA, 200 mM NaCl) to give a gradient of 5 concentrations (10, 17.5, 25, 32.5 and 40%). These sucrose solutions were layered in this order in a centrifugation tube (40PA, manufactured by Hitachi) and finally the partly digested DNA solution was layered on top of the gradient. After centrifuging at 20,000 rpm for 17 hours at 20°C by using a rotor SRP28 SA (manufactured by Hitachi), the mixture was divided into 80 portions (0.5 ml each) with a peristaltic pump to provide a fraction containing DNA fragments of 16 to 23 kb in the greatest amount. This DNA fraction was then ligated with a vector λDASH II/BamH (manufactured by STRATAGENE) by the action of T4 DNA ligase (manufactured by BOEHRINGER MENNHEIM) and then packaged into phage particles by using Gigapack II Gold packaging extract (manufactured by STRATAGENE). Thus, a rice genomic library was constructed, the size of which was calculated as about 5×10^6 pfu.

(2) Screening of clones

[0076] About 10,000 pfu of the phage was mixed with *E. coli* SRBP2 for infection and inoculated into a square Petri dish (14 x 10 cm). After an incubation at 39°C overnight, a nylon membrane filter Hybond N+ (manufactured by Amersham) was brought into contact with the plaque surface and then processed in accordance with the manufacturer's instructions attached to the filter. The probe was 0.6 kb EcoRI-SalI fragment in the 5'-side of the rice flower organ-specific cDNA (RPC213) which was used after being RI-labeled with the use of Multiprime Labeling System (manufactured by Amersham). Thus, plaque hybridization was carried out. The hybridization and washing were effected under the same conditions as those specified in the above Example 1(3) provided that 1 x Denhart's solution and carrier DNAs were not employed. From 100,000 plaques, 6 positive clones were thus selected. Next, phage DNAs were prepared from these plaques. They served as templates in the PCR which was performed with the use of the RPC213-specific primers 213S and 213AS. As a result, the expected product of about 250 bp was found to have been amplified in 2 clones named RPG106 and RPG107.

(3) Subcloning of region containing promoter

[0077] DNA was extracted from the above-mentioned 2 RPC213 genomic clones, digested with restriction enzymes

SacI and HindIII (manufactured by Takara Shuzo Co., Ltd.) and then the DNA fragments were fractionated in a 0.8% agarose gel. Also, DNA was isolated and purified by the phenol-SDS method (Komari et al. Theor. Appl. Genet. 77, 547-552, 1989) from paddy rice plants of varieties "Akihikari" and "IR24" about 1 month after sawing. About 5 μg of DNA was digested with SacI and HindIII and electrophoresed similar to the above case. Next, it was blotted onto a nylon membrane filter Hybond-N+ (manufactured by Amersham) and Southern hybridization was performed by using as a probe the above-mentioned cDNA fragment of 0.6 kb having been RI-labeled as in Example 1(4)1).

[0078] Hybridization and washing were carried out in accordance with the manufacturer's instructions attached to the filter. As a result, a band of the same size as the total genomic DNA appeared in RPG106. Thus, the SacI fragment (6.0 kb) of RPG106 reacting with the probe was subcloned into the same site of pBluescript. Next, restriction maps (Fig. 3) were formed by using 4 restriction enzymes (BglII, HindIII, SacI and SalI) to further specify the region containing the promoter. (4) Determination of whole nucleotide sequence of RPG106 SacI-SalI fragment (5.4 kb)

[0079] As Fig. 3 shows, the genomic clone RPG106 has four BglII sites. By using these restriction sites, RPG106 was first divided into five fragments. Namely, RPG106 SacI 6.0 kb (pBluescript) was digested with BamHI and BglII to give five fragments, i.e., SacI-BglII 0.7 kb (+pBluescript), BglII 2.1 kb, BglII 2.3 kb, BglII 0.8 kb and BglII-SacI 0.7 kb (multi-cloning site of +pBluescript). Then the nucleotide sequences of the former 4 fragments were determined. Since the SacI-BglII 0.7 kb fragment still contained pBluescript, this plasmid was cyclized again. Regarding the remaining 3 fragments, plasmids were constructed by inserting these fragments into the BamHI site of pBluescript in the regular direction and vice versa. The fragment BalII 2.1 kb had 2 SpeI sites and 1 XhoI site in it, while the fragment BglII 2.3 kb had 2 EcoRV site, 1 SalI site and 1 SpeI site. Each of these fragments was further subcloned by using these restriction sites to give 14 plasmids in total which covered almost the entire RPG106 SacI 6.0 kb. The nucleotide sequences of both strands of each of these plasmids were determined by using M13 primer (manufactured by Takara Shuzo Co., Ltd.) with Fluorescence Sequencer (Model 373A, manufactured by Applied Biosystems). The nucleotide sequences in regions which could not be decoded by this method were determined by the primer walking method and thus the entire nucleotide sequence of RPG106 SacI-SalI 5.4 kb (total nucleotide sequences 5396 bp) was determined. This nucleotide sequence is represented by SEQ ID NO:2 in Sequence Listing.

[0080] The sequence represented by SEQ ID NO:2 has the following characteristics.

nt1-nt5369, nt3335-nt5108 : sequences having been confirmed as having promoter activity by GUS.
nt4964-nt4969 : TATA box-like sequence.
nt4996, 4996, 4997 : transcription initiation points of RPC213 gene determined by the primer extension method.
nt5016-nt5018 : first initiation codon of RPC 213 gene.
nt5370-nt5372 : second initiation codon of RPC 213 gene.
nt5162-nt5242 : intron sequence.
nt1-nt6 : restriction enzyme SacI site.
nt792-nt734, nt2811-nt2816, nt5103-nt5108 : restriction enzyme BglII sites.
nt3335-nt3340 : restriction enzyme HindIII site.

[0081] Comparison of the RPC213 gene with the nucleotide sequence of RPC213 cDNA indicated that an intron sequence of 81 bp was located between the first ATG and the second ATG in the RPC213 gene. As shown in the shaded parts in Fig. 3, the nucleotide sequence in the region of about 300 bp from the 5'-terminus to the first SalI site in the cDNA completely agreed with the nucleotide sequence of the genomic DNA RPG106 corresponding to this region except the intron sequence.

(5) Determination of transcription initiation points

[0082] To specify the promoter region of RPC213, first, the 5'-terminus of the transcription unit was analyzed by RT-PCR. By reference to nucleotide sequence of the 3'-terminal region (300 bp) of the above-mentioned 2.3 kb BglII fragment of genomic clone RPG106, 4 sense primers (213A, 213B, 213C and 213D) and 1 antisense primer (213Z) were synthesized (Fig. 4). 10 ng of mature pistil cDNA was employed as a template, while 10 ng of leaf cDNA and 10 ng of genomic clone RPG106 BglII 2.3 kb fragment were employed as control. PCR was carried out under the same conditions as employed in Example 1 (4)2). As a result, the control leaf cDNA gave no amplification product in any combination of the primers. In contrast, pistil cDNA showed the amplification product of the same size as the genomic clone when primers 213A and 213Z were combined. Based on these results, it was considered that a split point (namely, a transcription initiation point or the 3'-terminus of intron) would be located between 213A and 213B.

[0083] Next, transcription initiation points were determined by the primer extension method. First, the primer employed in the RT-PCR:

213Z : 5'-TGCTGGTATGGATGTGATG-3' (SEQ ID NO:5);

and an additional primer for the primer extension experiment:

213Z-2: 5'-CTGACGAGGCTGTTGCTG-3' (Fig. 4)(SEQ ID NO:6);

were synthesized. These primers (10 pmole each) were RI-labeled at the 5'-terminus with the use of [γ-^{32}P]ATP according to the manufacturer's instructions attached to MEGARABEL Kit (manufactured by Takara Shuzo CO., Ltd.). 0.1 pmol (0.3×10^6 cpm) of these labeled primers and 50 μl of the total RNA of either immature spikelet (1 to 2 weeks before earing) or leaf were annealed in the presence of 3U/μl of RNase inhibitor (RNAguard, manufactured by Pharmacia) in a buffer (0.25 M KCl, 2 mM Tris-HCl pH 8.0, 0.2 mM EDTA) in a reaction system of 10 μl at 42°C for 2 hours. After adding 30 μl of another buffer (66 mM Tris-HCl pH 8.3, 6.6 mM $MgCl_2$, 1.3 mM DTT, 0.66 mM dNTP, 130 μg/ml actinomycin D) and 1 μl (200 units) of a reverse transcriptase (SUPERSCRIPT, manufactured by BRL), the mixture was maintained at 42°C for 1 hour. Then ethanol and ammonium acetate were added to allow precipitation to occur. After washing the precipitate with 70% ethanol, the product was air-dried and then dissolved in an electrophoresis buffer which was prepared by mixing the reaction termination solution of T7 Sequencing Kit (manufactured by Pharmacia) with 0.1 M NaOH containing 1 mM EDTA (2 : 1).

[0084] Then the whole solution was heated at 95°C for 3 minutes and then electrophoresed on a 6% agarose gel. By using the same primers, a sequencing reaction was carried out with T7 Sequencing Kit by using a plasmid containing RPG106 BglII 2.3 kb fragment as a template. Then the product thus obtained and the 10 bp and 50 bp ladders (manufactured by BRL), which had been RI-labeled at the terminus via an exchange reaction with the use of [γ-^{32}P]ATP according to the manufacturer's instructions attached to MEGARABEL kit (manufactured by Takara Shuzo Co., Ltd.), were electrophoresed simultaneously. The results are shown in Fig. 5. No extension product was obtained from leaf RNA in which the gene was probably not expressed, while 2 bands (in the case of the 213Z primer) and 3 bands (in the case of the 213Z-2 primer) of extension products were detected by using the total RNA of immature spikelet as the template. Comparison with the sequence ladders electrophoresed side by side indicated that the products by these primers were detected at the same position. These results indicated that 3 consecutive transcription initiation nucleotides "CAA" were located between 213A and 213B and the transcription of RPC213 was initiated from the cytosine or adenines. As Fig. 4 clearly shows, a TATA box-like sequence (5'-TATAAAT-3') was located 31 bp upstream of the C (cytosine) of the most upstream transcription initiation point. The distance between this TATA box and the transcription initiation point coincided with genes of other plants (Joshi, Nucleic Acids Res., 156, 6643-6653, 1987). Further, there was an initiation codon (the first ATG) 21 bp downstream of the C of the transcription initiation point. Since the reading frame containing this ATG agreed with the reading frame of the cDNA as described above, it is generally considered that the ATG 21 bp downstream of the transcription initiation point would be the initiation codon. However, it is also considered that the distance between the transcription initiation point and the initiation codon is too short. Accordingly, there is a possibility that the second ATG located 273 bp downstream of the first ATG in the same reading frame might be the actual initiation codon. Moreover, C (cytosine) was located 3 nucleotides upstream of A (adenine) in the first ATG. In contrast, A (adenine) was located 3 nucleotides upstream of A (adenine) in the second ATG, which well agreed with the consensus in nucleotides around the initiation codon of mRNA in eucaryotic cells (Kozak, J. Cell Biol., 108, 229-241, 1989).

Example 3: Analysis of promoter expression site

(1) Construction of vectors for analyzing promoter expression and transformation of rice

[0085] To analyze the expression of the isolated promoter *in vivo,* vectors having GUS reporter gene linked thereto were constructed in the following manner (Fig. 6). The vector used in this example was pSB21 (Komari et al. Plant J., 10, 165-174, 1996). Use was made of the unique HindIII site and BamHI site located at each termini of 35S promoter contained in this vector.

[0086] First, RPC106 SacI 6.0 kbp (pBluescript) was co-digested with HindIII and BglII to isolate a promoter fragment of about 1.8 kbp from the region which precedes the BglII site located 87 bp downstream of the first ATG in the RPC213 gene. This fragment was ligated to vector pSB21 having been digested with the same enzymes to delete the 35S promoter therefrom. The obtained plasmid vector was named pYOT213αG. In pYOT213αG, the first ATG of RPC213 gene and the ATG of the GUS gene were contained in the same reading frame. Therefore, when the translation of the RPC213 gene was initiated from the first ATG, the GUS protein would be translated as a fusion protein.

[0087] Second, considering the possibility that the translation of the RPC213 gene might be initiated from the second ATG, another vector was constructed in the following manner to isolate a promoter fragment from a broader region. To amplify a part of the promoter region by PCR, a pair of primers:

213P-5H-2 : 5'-GACGTGATCCACGGCATTGACG-3' (SEQ ID NO;7),
213P 2ndATG-Bam: 5'-CGGGGATCCGTTCTCCTCCACCCACGC-3' (SEQ ID NO:8);

were synthesized. 213P-5H-2 matches a region upstream of the unique HindIII site. 213P 2ndATG-Bam matches the nucleotide sequence immediately upstream of the second initiation codon ATG and has a BamHI site. PCR was performed in a reaction system of 100 μl by using these primers (100 pmole each), about 1 μg of DNA (alkali-denatured template RPG106) and Extaq (manufactured by Takara Shuzo Co., Ltd.).

[0088] The reaction mixture was subjected to PCR consisting of 3 minutes at 94°C for 1 cycle; 1 minute at 94°C, 1 minute at 60°C and 2.5 minutes at 72°C for 20 cycles; and 6 minutes at 72°C for 1 cycle. The amplification product was cloned into pCRII (manufactured by Invitrogen) and then the nucleotide sequence was confirmed. This plasmid was digested with HindIII and BamHI and the RPC213 promoter fragment of 2.0 kb was isolated therefrom. Next, the fragment was ligated to the vector pSB21 having been treated by the same enzymes to delete the 35S promoter. The plasmid thus obtained was further digested with HindIII and dephosphorylated. Next, an RPG106 HindIII fragment of 3.3 kb, which was obtained by digesting RPG106 SacI 6.0kb (pBluescript) with HindIII, was inserted thereinto. The resultant plasmid vector was named pYOT213βG. In this vector, a GUS gene is located downstream of the promoter fragment, and the promoter fragment is composed of about 5.3 kb upstream region from the second ATG of the RPC213 gene. The two vectors thus constructed were each transferred into *Agrobacterium tumefaciens* LBA4404 by tri-parental mating and used in an experiment of the transformation of rice.

[0089] The transformation of rice was carried out by using calli developed from immature rice embryo of "Tsukinohikari" in accordance with the method of Hiei et al. (Plant J., 6, 271-282, 1994).

(2) Analysis of promoter expression site by way of histological observation of GUS

[0090] According to the method of Jefferson et al. (EMBO J., 6, 3901-3907, 1987), various organs (leaf, root, spikelet in earing and spikelet in flowering) of the rice, transfected with pYOT213αG or pYOT213βG, were GUS-stained with the use of X-gluc. (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) as the substrate in order to histologically observe the cells under a stereoscopic microscope as well as an optical microscope. Observation was made on organs in spikelet i.e. pistil, anther, lodicule, palea and lemma and spikelet base. The GUS expression level by the promoter was evaluated in 4 grades from "strong (++)" to "less than detection limit (-)" (Table 2). In Fig. 7, black bars stand for "strong expression", shaded bars stand for "moderate to weak expression", dotted bars stand for "very weak expression" and white bars stand for "no expression", while L, R, P, A, RO, G and BS respectively stand for leaf, root, pistil, anther, lodicule, palea and lemma, and spikelet base.

[0091] As a result, many individuals transfected with pYOT213αG were not stained with GUS in any organ examined. However, 5 individuals showed the expression of the GUS gene by the promoter activity in at least one of the organs. Among these individuals, pYOT213αG-17 showed GUS expression in pistil and lodicule and very weak expression in palea and lemma and spikelet base (Table 2). These results generally agreed with the results of Northern hybridization and RT-PCR. pYOT213αG-4 showed the GUS expression specifically in pistil (Table 2). The expression in pistil was observed around the border of stigma and ovary (Fig. 8A). None of these 5 individuals showed the GUS expression in leaf, root and anther. 2 individuals showed the expression in pistil, one showed in lodicule (Fig. 7). In addition, very weak expression was observed in palea and lemma in 4 individuals and in spikelet base in 3 individuals.

TABLE 2

Table 2: Analysis of GUS expression site by 213 promoter

Vector	Plant No.	Organ of rice transformant						
		leaf	root	pistil	anther	lodicule	palea/lemma	spikelet base
pYOT213αG	4	-	-	+	-	-	-	-
	6	-	-	-	-	-	±	-
	17	-	-	+	-	+	±	±
	23	-	-	-	-	-	±	±
	28	-	-	-	-	-	±	±

TABLE 2 (continued)

Table 2: Analysis of GUS expression site by 213 promoter

Vector	Plant No.	Organ of rice transformant						
		leaf	root	pistil	anther	lodicule	palea/lemma	spikelet base
pYOT213βG	3	±	±	+	+	-	++	+
	4	+	+	+	+	++	++	+
	6	+	+	++	±	±	+	±
	7	±	±	+	±	++	++	+
	8	-	-	+	-	+	+	±
	11	+	+	+	+	++	++	++
	13	+	+	+	-	+	+	+
	15	+	±	+	±	-	+	-
	16	±	±	+	±	-	±	-
	17	±	-	+	-	-	±	+
	20	+	-	+	-	+	+	-
	22	+	±	+	+	++	++	++
	23	+	-	+	-	++	++	-

++: strong expression; +: moderate to weak expression; ±: little expression; and -: no expression.

[0092] On the other hand, 5 individuals (pYOT213βG-3, 7, 8, 16 and 17) among 13 individuals transfected with pYOT213βG showed no or very weak GUS expression in leaf and root, and showed the GUS expression in flower organ, namely, they showed the flower organ-specific promoter activity (Table 2). Among them, 2 individuals (pYOT213βG-7 and 8) presented results well agreeing the results of Northern hybridization and RT-PCR, i.e., relatively strong GUS expression in pistil and lodicule and very weak expression in anther. The other eight transformants than those showing the flower organ-specific expression also showed relatively strong promoter activity in flower organs even though they showed the GUS expression in leaf and root (Table 2). For example, pYOT213βG-6, which showed the GUS expression in leaf and root, exhibited stronger expression in pistil than in these organs. In this individual, weak expression was also observed in palea and lemma, anther, lodicule and spikelet base (Fig. 8B). pYOT213βG-17 showed very weak or no expression in leaf and root (Fig. 8D) but relatively strong expression in pistil (Fig. 8C). In pistil of the individuals transfected with pYOT213βG, the GUS expression was observed mainly in the stigma, i.e., stigma axis and hairy tissues in stigma (Fig. 8B and C). The results of the examination of the GUS expression in the organs are summarized in Fig. 7, which indicated that no individual showed strong expression in leaf or root. About 1/2 or more of the individuals showed moderate to weak expression in leaf, while about 1/3 or less of the individuals showed moderate to weak expression in root. The remaining individuals showed very weak or no expression in leaf or root. In flower organs, in contrast, strong promoter activity was observed in all organs except anther (i.e., pistil, lodicule, palea and lemma and spikelet base). In particular, all of the 13 individuals showed definite GUS expression in pistil and one of them showed an intensely blue GUS-stain, thus indicating strong expression. In lodicule and palea and lemma, the expression of the GUS gene by the promoter activity was observed in about 2/3 of the all individuals and more than 1/2 thereof (5 individuals for lodicule and 6 individuals for palea and lemma) showed strong GUS expression. Also, 2 individuals showed strong expression in spikelet base.

[0093] Based on these results, it has been clarified that these two DNA fragments ligated to the GUS gene have promoter activities predominant in flower organs. It is also found that 213β having a longer fragment has the stronger activity. Since these promoter fragments are similar with each other in organ-specificity even though the promoter activity of 213β is higher than that of 213α, it is expected that a nucleotide sequence regulating the expression level (contributing to enhanced expression) of the RPC213 promoter will be contained in the SacI-HindIII 3.3 kb fragment in the 5'-side from 213α, which is contained in the nucleotide sequence of 213β but not in the nucleotide sequence of 213α, or in the DNA sequence from BglII to the second ATG in the 3'-side from 213α. The latter sequence is seemingly the more likely candidate.

INDUSTRIAL APPLICABILITY

[0094] The present invention makes it possible to genetically manipulate flower organs such as pistil and lodicule.

SEQUENCE LISTING

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<130> YCT-378

<150> JP Application No. Heisei 10-43372

<151> 1998-2-25

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Thr Ala Ser Ser Ala Trp Leu Cys Cys Pro Asn His His Ile His Thr
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 75                  80                  85                  90
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Asn Met Leu Pro Leu Leu Thr Pro Val Asp Ser Ala Trp Gln Pro His
                 95                 100                 105
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Asp Phe Leu Pro Cys Ser Ala Ala Gly Gly Gly Glu Ala Leu Ala Ala
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Phe Thr Glu Gly Val Ala Glu Leu Arg Ala Gly Ala Ala Gly Val Pro
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GAC GAG GTG CTG GTC TGC CTC GTG GGG AAC ATG GTG ACG GAG GAG GCG    483
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    140                 145                 150
CTC CCG ACG TAC CAG AGC ATG GGC AAC CGC GCC GAG GGC CTC GCC GAC    531
Leu Pro Thr Tyr Gln Ser Met Gly Asn Arg Ala Glu Gly Leu Ala Asp
155                 160                 165                 170
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Gly Thr Gly Val Ser Pro Leu Pro Trp Ala Arg Trp Leu Arg Gly Trp
                175                 180                 185
ACC GCC GAG GAG AAC CGC CAC GGC GAC CTC CTC AAC CGC TAC CTC TAC    627
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Claims

1. A DNA fragment comprising the sequence of positions 3335 to 5108 in the nucleotide sequence represented by

SEQ ID NO:2 in Sequence Listing, a part of said sequence or a sequence derived from said sequence by deletion, substitution, insertion or addition of one or more nucleotides and having a promoter activity.

2. A DNA fragment as claimed in Claim 1, wherein a part of the sequence of positions 3335 to 5108 in said nucleotide sequence represented by SEQ ID NO:2 is located downstream of the point of at least 500 nucleotides upstream of the transcription initiation points (the nucleotides of positions 4995 to 4997 in said nucleotide sequence represented by SEQ ID NO:2).

3. A DNA fragment as claimed in Claim 1, wherein a part of the sequence of positions 3335 to 5108 in said nucleotide sequence represented by SEQ ID NO:2 is located in the region upstream of the first initiation codon (the nucleotides of positions 5016 to 5018 in said nucleotide sequence represented by SEQ ID NO:2).

4. A DNA fragment as claimed in Claim 3, wherein said region upstream of the initiation codon is located downstream of the point of at least 500 nucleotides upstream of the transcription initiation points.

5. A DNA fragment as claimed in Claim 1, wherein a part of the sequence of positions 3335 to 5108 in said nucleotide sequence represented by SEQ ID NO:2 is located in the region upstream of the transcription initiation points (the nucleotides of positions 4995 to 4997 in said nucleotide sequence represented by SEQ ID NO:2).

6. A DNA fragment as claimed in Claim 5, wherein said region upstream of the transcription initiation points is the region of at least 500 nucleotides upstream of the transcription initiation points.

7. A DNA fragment comprising the sequence of positions 3335 to 5108 in the nucleotide sequence represented by SEQ ID NO:2 in Sequence Listing or a sequence derived from said sequence by deletion, substitution, insertion or addition of one or more nucleotides and having a promoter activity.

8. A DNA fragment comprising the sequence of positions 1 to 5369 in the nucleotide sequence represented by SEQ ID NO:2 in Sequence Listing, a part of said sequence or a sequence derived from said sequence by deletion, substitution, insertion or addition of one or more nucleotides and having a promoter activity.

9. A DNA fragment as claimed in Claim 8, wherein a part of the sequence of positions 1 to 5369 in said nucleotide sequence represented by SEQ ID NO:2 is located downstream of the HindIII site (the nucleotides of positions 3335 to 3340 in said nucleotide sequence represented by SEQ ID NO:2).

10. A DNA fragment as claimed in Claim 8, wherein a part of the sequence of positions 1 to 5369 in said nucleotide sequence represented by SEQ ID NO:2 is located downstream of the point of at least 500 nucleotides upstream of the transcription initiation points (the nucleotides of positions 4995 to 4997 in said nucleotide sequence represented by SEQ ID NO:2).

11. A DNA fragment as claimed in Claim 8, wherein a part of the sequence of positions 1 to 5369 in said nucleotide sequence represented by SEQ ID NO:2 is located upstream of the third BglII site (the nucleotides of positions 5103 to 5108 in said nucleotide sequence represented by SEQ ID NO:2).

12. A DNA fragment as claimed in Claim 8, wherein a part of the sequence of positions 1 to 5369 in said nucleotide sequence represented by SEQ ID NO:2 is located in the region upstream of the first initiation codon (the nucleotides of positions 5016 to 5018 in said nucleotide sequence represented by SEQ ID NO:2).

13. A DNA fragment as claimed in Claim 8, wherein a part of the sequence of positions 1 to 5369 in said nucleotide sequence represented by SEQ ID NO:2 is located upstream of transcription initiation points (the nucleotides of positions 4995 to 4997 in said nucleotide sequence represented by SEQ ID NO:2).

14. A DNA fragment comprising the sequence of positions 1 to 5369 in the nucleotide sequence represented by SEQ ID NO:2 in Sequence Listing or a sequence derived from said sequence by deletion, substitution, insertion or addition of one or more nucleotides and having a promoter activity.

15. A chimeric DNA sequence comprising a DNA fragment having a promoter activity as claimed in any of Claims 1 to 14 and a desired structural gene under control of the promoter acitivty.

16. A transformation vector having a chimeric DNA sequence as claimed in Claim 15.

17. A DNA fragment having a flower organ-specific promoter activity which is hybridizable with the sequence of positions 1 to 5369 in the nucleotide sequence represented by SEQ ID NO:2 in Sequence Listing or a part of said sequence having a flower organ-specific promoter activity.

18. A DNA fragment as claimed in Claim 17, wherein said hybridization is performed under conditions with a moderate hybridization intensity.

19. A flower organ-specific promoter sequence which can be identified from among DNA sequences obtained by screening a genomic library of rice or another plant by using as a probe the nucleotide sequence represented by SEQ ID NO:1 in Sequence Listing or a part of said sequence.

20. A flower organ-specific promoter sequence as claimed in Claim 19, wherein said screening is performed via hybridization under conditions with a moderate hybridization intensity.

21. A DNA fragment comprising a sequence having at least 15 consecutive nucleotides in the sequence from positions 22 to 1278 of the sequence represented by SEQ ID NO:1 in Sequence Listing or a nucleotide sequence complementary to said sequence.

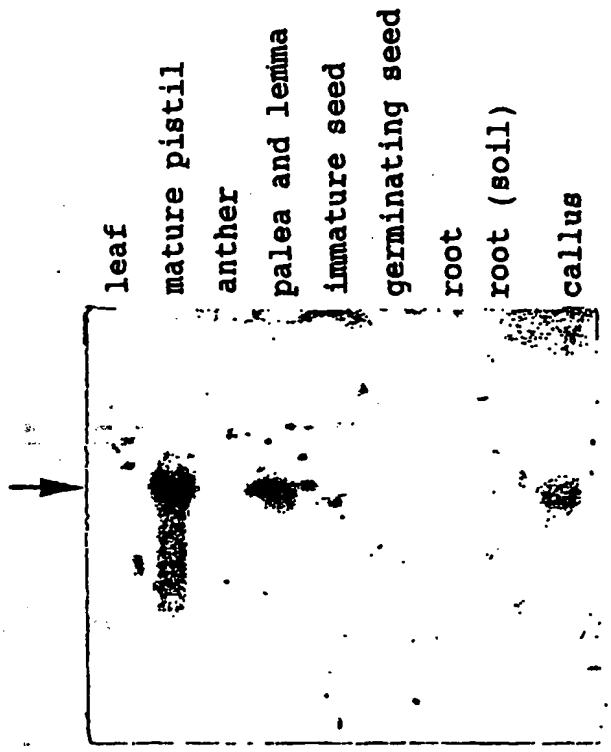
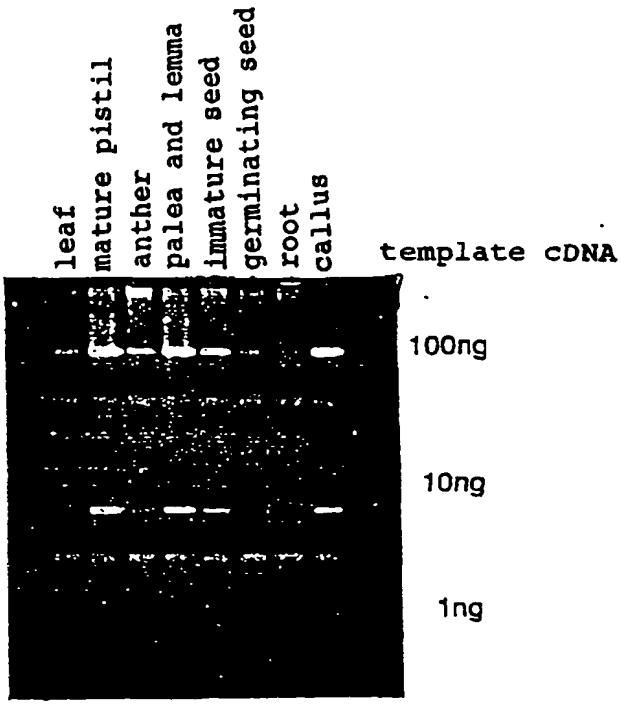


Fig. 1

Northern hybridization analysis of RPC213 gene



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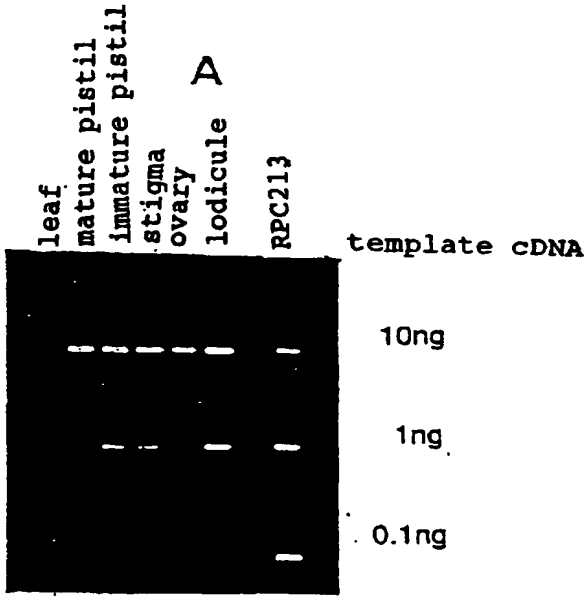

B

Fig. 2

Reverse transcription PCR analysis of RPC213

When 1 ng of template cDNA was used (A), faint bands were observed in the lanes of mature pistil and palea and lemma (not clear in the above photograph).

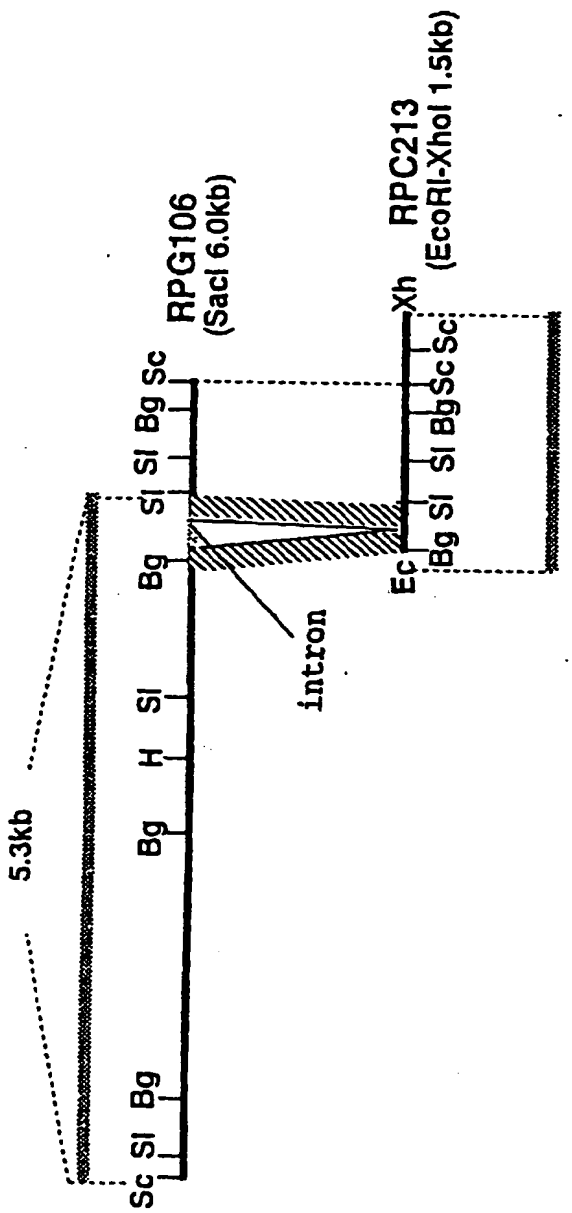

Fig. 3

Comparison of restriction maps of RPG106 and RPC213

Bg: BglII, Ec: EcoRI, H: HindIII, Sc: SacI, Sl: SalI, Xh: XhoI

Dotted part indicates a region with identified nucleotide sequence, while shaded parts indicate regions having the same nucleotide sequence.

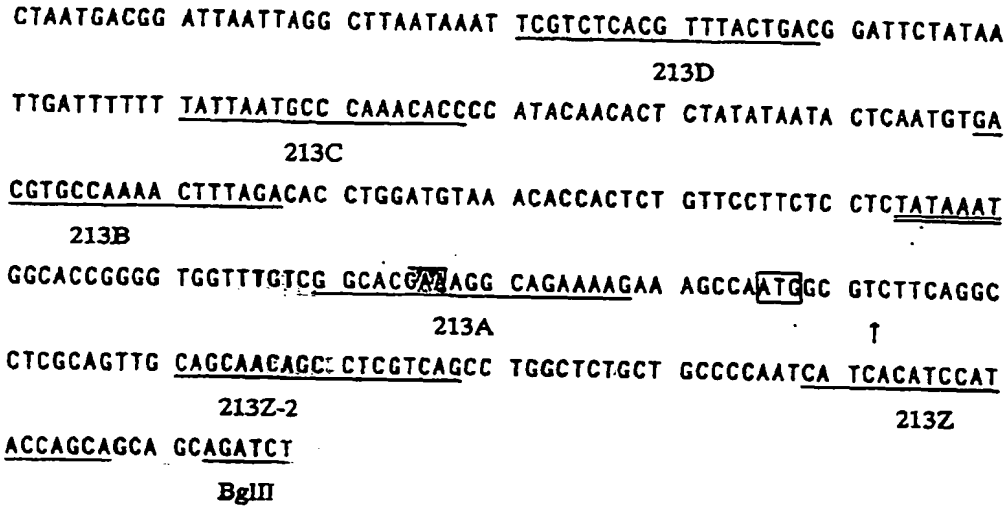

Fig. 4

Nucleotide sequence around transcription initiation point of RPC213

Nucleotide sequence of 3′-region of RPG106BglII 2.3 kb. Primers employed in RT-PCR analysis and primer extension analysis are underlined. Arrow shows the 5′-terminus (C) of RPC213. Transcription initiation points are shaded, TATA box-like sequence is double-underlined, and the first initiation codon is framed.

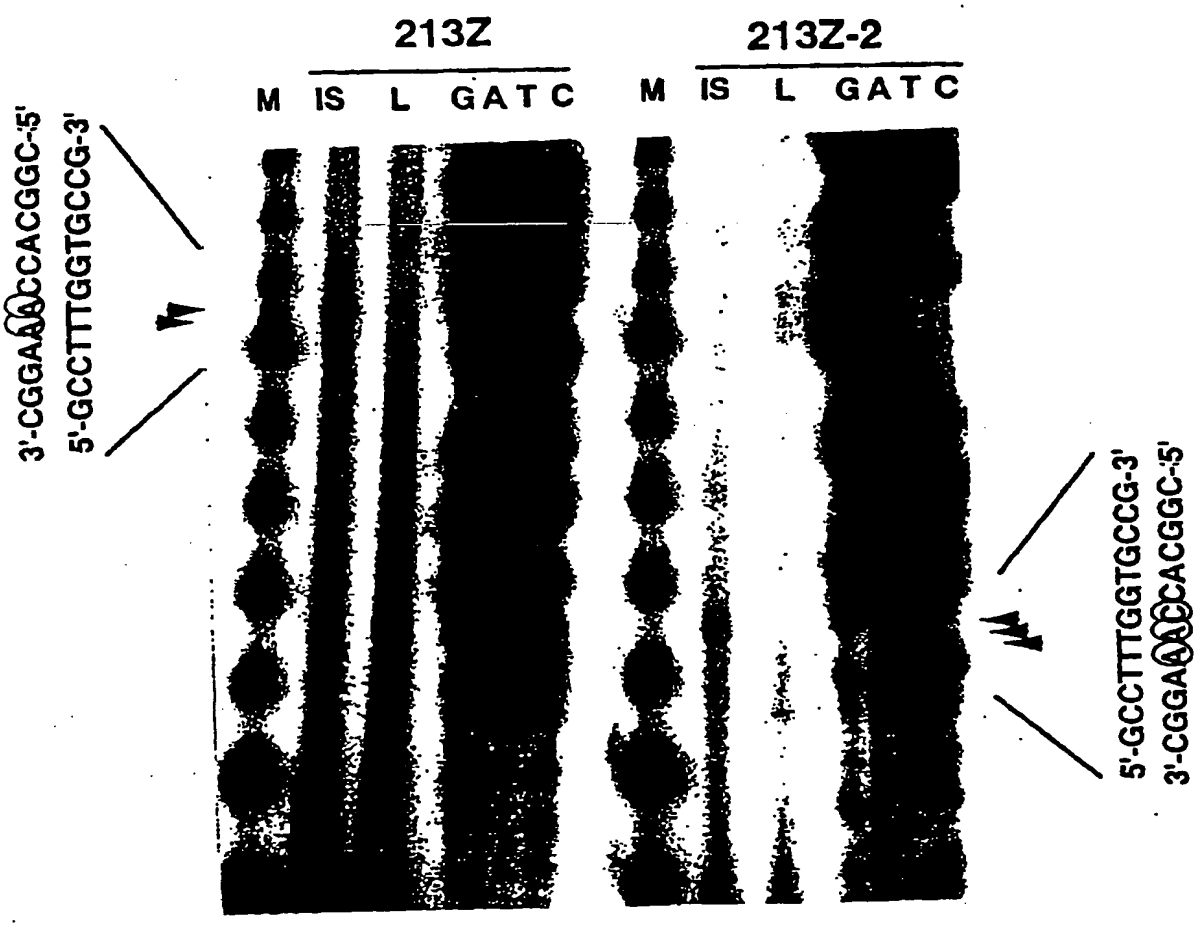

Fig. 5

Determination of transcription initiation points of RPC213

M: 10 bp, 50 bp ladders, IS: immature spikelet,

L: leaf.

Arrows: primer extension products (IS lane).

Nucleotide sequences decoded from sequence ladders are given in both sides and transcription initiation points are circled.

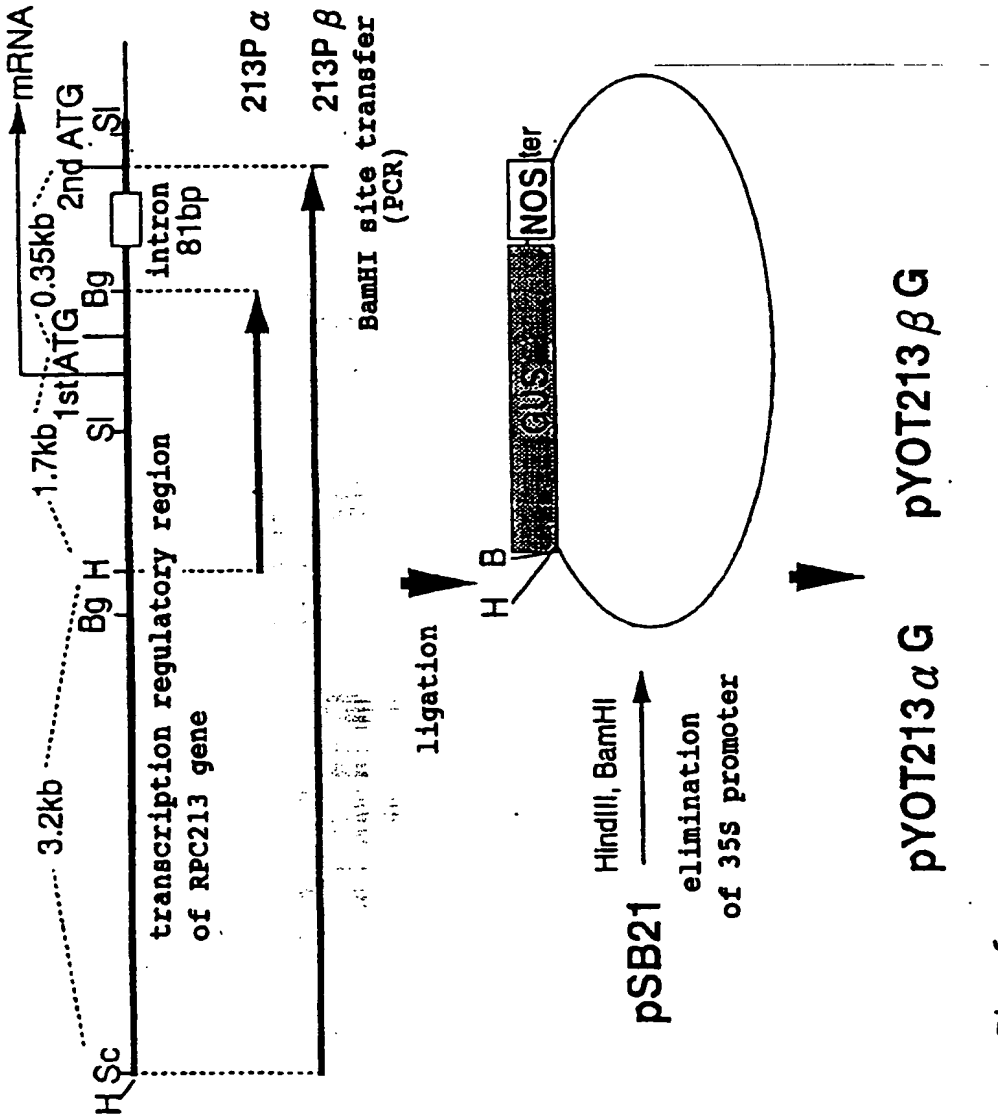

Fig. 6

Construction of vectors for analyzing promoter expression

B: BamHI, Bg: BglII, H: HindIII, Sc: SacI, Sl: SalI.

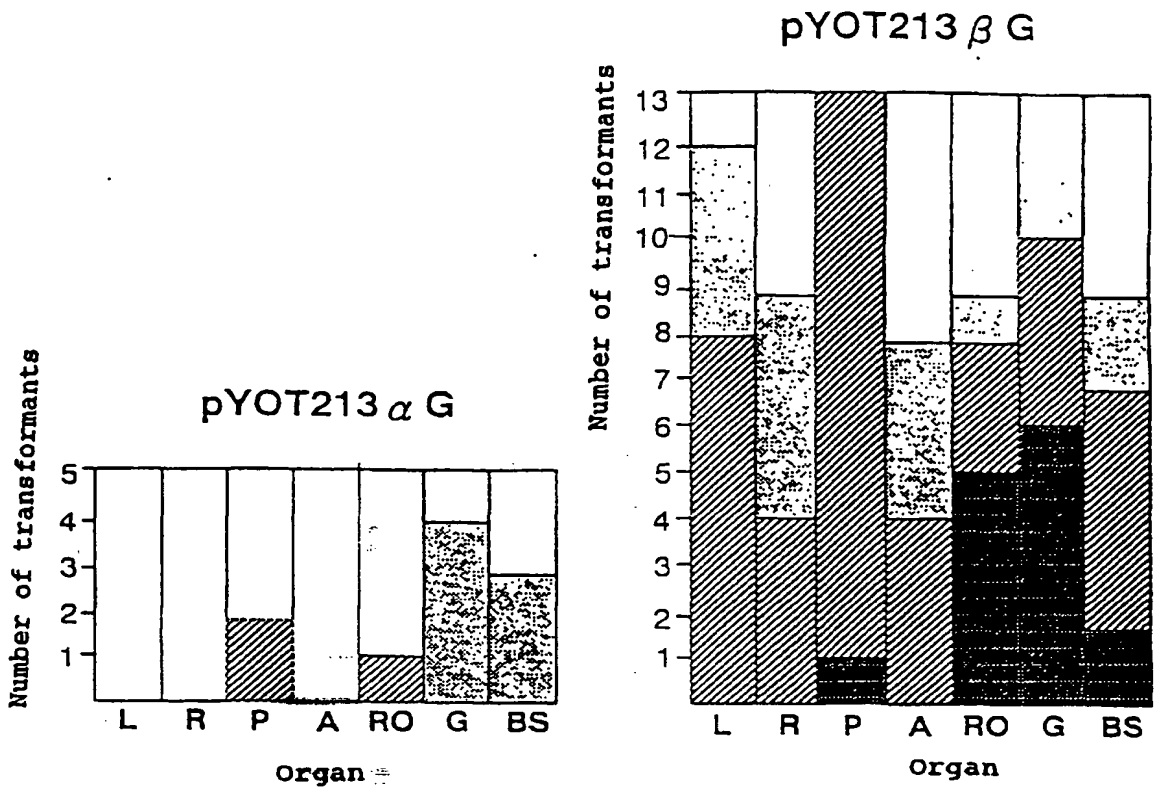

Fig. 7

Analysis of expression of 213 promoter in various organs

Formed on the basis of GUS expression analysis data given in Table 2.

The abscissa refers to the organs of the transformants used in examining the GUS expression while the ordinate refers to the number of individuals showing the expression.

■: strong expression ▨: moderate to weak expression

▩: very weak expression and □: no expression

L: leaf, R: root, P: pistil, A: anther,
RO: lodicule, G: palea and lemma, and BS: spikelet base.

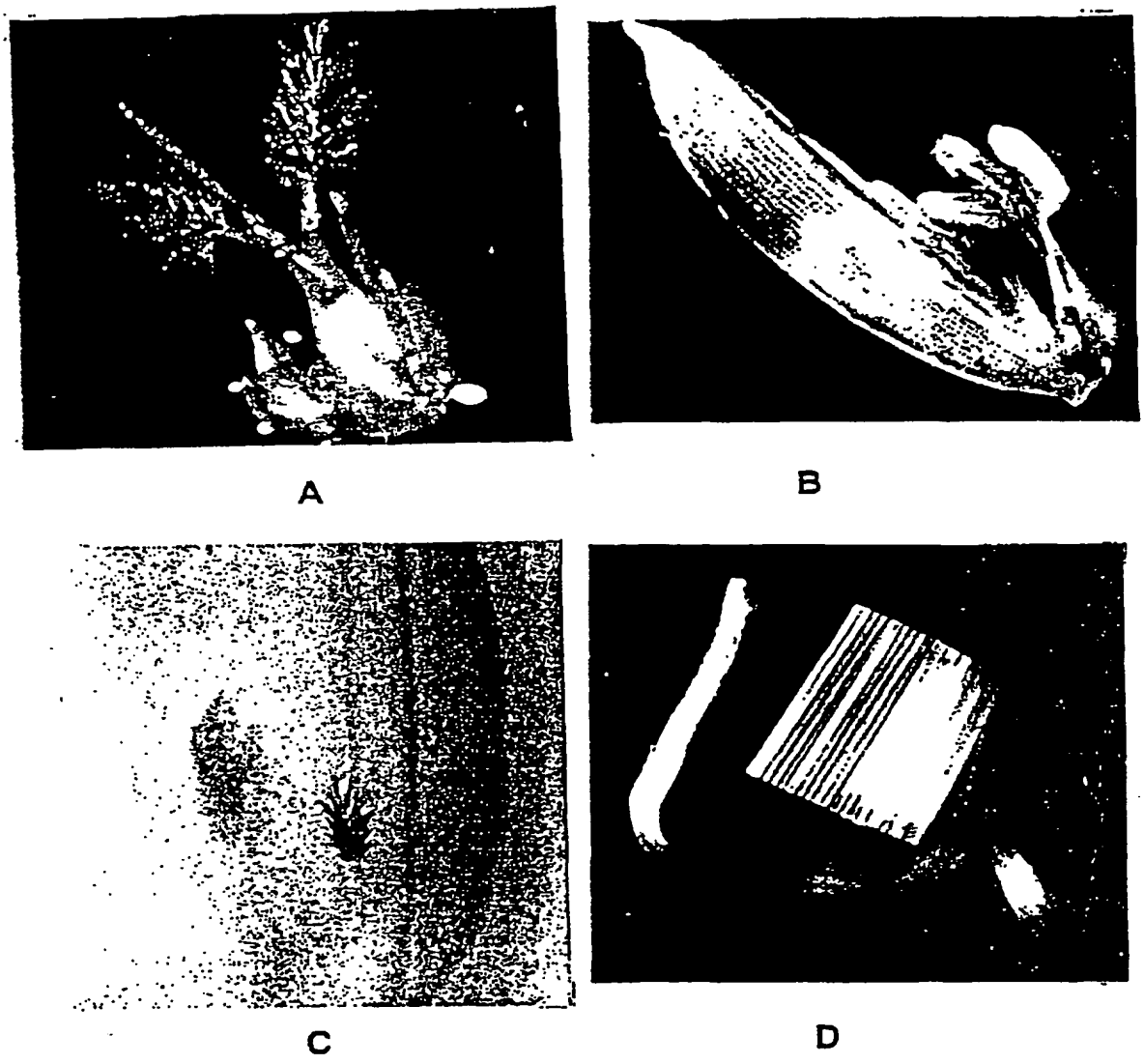

Fig. 8

Analysis of GUS expression by 213 promoter

A: expression of 213αG-4 in pistil.

B: expression of 213βG-6 in flower organ.

C: expression of 213βG-17 in pistil.

D: expression of 213βG-17 in leaf and root.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP99/00568

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[6] C12N15/29, 15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[6] C12N15/29, 15/82

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
GenBank/EMBL/DDBJ, WPI (DIALOG), BIOSIS (DIALOG)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Genomics, Volume 10, Number 4, issued 1991, Werner Albig et al., "Isolation and Characterization of Two Human H1 Histone Genes within Clusters of Core Histone Genes", pages 940-948	21
X	Journal of Virology, Volume 70, Number 1, issued January 1996, Tai-An Cha et al., "Human Cytomegalovirus Clinical Isolates Carry at Least 19 Genes Not Found in Laboratory Strains", pages 78-83 & Database GenBank, Accession No. U33331	21
X	Database GenBank, Accession No. U91339, August 9, 1997, Hongtrakul, V. et al., "Helianthus annuus stearoyl-ACP desaturase mRNA, complete cds."	21
PX	Database GenBank, Accession No. AU029718, October 19, 1998, Sasaki, T. and Yamamoto, K., "Rice panicle shorter than 3cm Oryza sativa cDNA clone E31372_6Z, mRNA sequence."	21

[x] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
11 May, 1999 (11. 05. 99)	25 May, 1999 (25. 05. 99)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

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